REMARKS

Applicants appreciate the examination of the present application as evidenced by the Office Action dated December 8, 2010 (hereinafter, "the Office Action"). Upon entry of this Amendment, Claims 1, 3, 6-10, 12-16, 25, 28 and 31-39 are pending in the present application, and these claims stand rejected. Claim 14 has been canceled herein without prejudice.

In view of the foregoing amendments and following remarks to address the issues raised in the Office Action, reconsideration and withdrawal of the rejections to the present application are respectfully requested, and favorable action upon all pending claims is hereby requested.

I. Claim Rejections Under 35 U.S.C. §103

Claims 1, 3, 6-10, 12-16, 25, 28 and 31-39 stand rejected under 35 U.S.C. §103(a) as allegedly being unpatentable over Gerba et al., "Endotoxin Removal by Charge-Modified Filters," *Applied and Environmental Microbiology*, Dec. 1985, p. 1375-1377 (hereinafter, "Gerba") in view of Brown et al., "The distribution of infectivity in blood components and plasma derivatives in experimental models of transmissible spongiform encephalophathy," *Transfusion*, Sept. 1998, p. 810-816 (hereinafter, "Brown"). *See*, Office Action, page 3. Specifically, the Examiner contends that "it would have been prima facie obvious to provide the methods of removal of infectious prions from plasma taught by Brown using Gerba's depth filter comprising cellulose and kieselguhr because Gerba teaches that his depth filter allows for enhanced removal of bacteria and bacterial endotoxin from solution." Office Action, page 4. However, Applicants respectfully disagree and submit that Gerba, alone or in combination with Brown, fails to render the pending claims obvious.

As an initial matter, Applicants respectfully submit that Gerba is directed to a completely different biological entity than that of the claimed invention. Gerba is directed to the removal of endotoxins, which, as provided by Gerba, are "lipopolysaccharide components derived from the outer cell membranes of gram-negative bacteria and blue-green algae (cyanobacteria)." Gerba, page 1375, col. 1, paragraph 1. Lipopolysaccharides are combinations of lipids and sugars with a molecular formula of $C_{116}H_{214}O_{39}P_2$ and a molecular weight ratio of 2323. *See*, Qureshi et al., "Complete Structural Determination of Lipopolysaccharide Obtained from Deep Rough Mutant of *Eschericha coli*," *J. Biol. Chem.* (1998) 263, p. 11971-11976, p. 11971, col. 1, second paragraph, *copy enclosed*. Bacterial endotoxins can cause an inflammatory response, characterized by

pyrogenic reactions, when administered as a contaminant of parenteral products.

In contrast, prions are proteins comprising approximately 200 amino acids with a molecular weight ratio of 35000-36000. The formula of individual amino acids is $C_2H_4O_2NR$, where R is a group containing 1-9 carbon atoms, 1-11 hydrogen atoms, 0-3 nitrogen atoms, 0-1 sulphur atoms and 0-2 oxygen atoms, and the molecular weight ratio is in the range of 75 to 204. Native prion protein may function in the repair of myelin. However, the "infectious" prion form of concern modifies native prion protein causing deposition of prion plaques.

Accordingly, endotoxins are a completely different and distinct biological entity than prions that differ from each other in structure, function, and chemistry. Not only is there no teaching or suggestion in Gerba that prions are similar to endotoxins, but there also is no teaching or suggestion that prions would function similarly to endotoxins in the methods described in Gerba, as the Examiner alleges. Moreover, there is nothing in the size, chemistry, or function of endotoxins and prions that would suggest to one of ordinary skill in the art that prions and endotoxins would have similar filtration properties.

In addition, Gerba teaches away from the claimed invention. The removal of bacteria endotoxins by filtration, as described in Gerba, is limited to water or aqueous solutions containing small molecules (*e.g.*, glucose, sucrose). Gerba reports that the removal of endotoxins from depth filters was decreased in the presence of 5% newborn calf serum. *See*, Gerba, p. 1377, col. 1, second paragraph, and Table 2. Gerba states that proteins in the serum likely compete with the endotoxin for adsorptive sites on the filters and that "no significant removal of endotoxin was observed with negatively charged filter media." Gerba, Abstract. Thus, Gerba teaches that removal of bacterial endotoxins by filtration would not be effective when handling complex biological solutions of proteins, such as serum. Additionally, given that proteins in serum, such as serum albumin, are negatively charged, one skilled in the art would find that Gerba teaches away from the filtration of negatively charged liquids and complex biological solutions of proteins, such as serum. Accordingly, Applicants respectfully submit that the teachings of Gerba would lead one of ordinary skill in the art away from using the filtration methods described in Gerba with biological liquids of proteins, such as plasma.

As set forth in the "Examination Guidelines for Determining Obviousness Under 35 U.S.C. 103 in View of the Supreme Court Decision in *KSR International Co. v. Teleflex Inc.*"

("Examination Guidelines") at page 57529, third column, first full paragraph, and as stated in *KSR*, "[w]hen the prior art teaches away from combining certain known elements, discovery of successful means of combining them is more likely to be nonobvious." Furthermore, it is clearly established in the case law that a *prima face* case of obviousness can be rebutted by evidence "that the prior art teaches away from the claimed invention in any material respect." *In re Peterson*, 315 F.3d 1325, 1331 (Fed. Cir. 2003) (emphasis added). Such evidence of a teaching away is readily apparent in the disclosure of Gerba.

Gerba is directed to a completely different subject matter, *i.e.*, endotoxins, than that of the claimed invention and not only provides no teaching or suggestion to one of ordinary skill in the art to modify the method of Gerba to achieve the claimed invention, but also teaches away from the claimed invention. Accordingly, one of ordinary skill in the art would have come away from a reading of Gerba with no motivation to carry out the methods of the claimed invention or any reasonable expectation or prediction that such methods would work as recited in the claims. Thus, Applicants respectfully submit that it would not have been obvious to one of ordinary skill in the art to modify the teachings of Gerba to arrive at the claimed invention and one skilled in the art would be directed away from doing so.

Brown fails to correct the deficiencies of Gerba. Brown examines the infectivity in blood components and Cohn plasma fractions in normal human blood that was "spiked" with trypsinized cells from a scrapie-infected hamster brain and in blood of clinically ill mice inoculated with a mouse-adapted strain of human transmissible spongiform encephalopathy. Infectivity of prion proteins in various protein fractions was measured by injecting the blood specimens into healthy animals. Thus, Brown is directed towards an assessment of risk to patients receiving plasma products, not towards the planned and controlled removal of prion protein contaminants.

Brown fails to teach or suggest the use of filters or a filtration process. Rather, Brown describes a plasma fractionation process, which is a scaled-down approximation of the precipitation procedures used during manufacture of plasma products. Moreover, Brown fails to teach or suggest a reason to modify the filtration methods of Gerba, directed to the removal of endotoxins, to achieve the claimed invention. Furthermore, while Brown reported different levels of infectivity for the different plasma fractions, these findings allow a calculation of relative risk to humans receiving these plasma fractions, but offer no insight into the claimed invention. Thus, Brown fails to correct

the deficiencies of Gerba and to teach or suggest the claimed invention.

Applicants respectfully submit that the combination of cited references would not have provided one of ordinary skill in the art with a *reason* that would have prompted such a person to combine the elements *in the manner recited in the pending claims*. Instead, Gerba is directed to different subject matter than that of the claimed invention and teaches away from the claimed invention, and Brown fails to cure these deficiencies. Accordingly, Applicants respectfully request that the rejection of claims 1, 3, 6-10, 12-16, 25, 28 and 31-39 under 35 U.S.C. §103(a) be withdrawn.

II. Claim Rejections Under 35 U.S.C. §102

Claim 14 stands rejected under 35 U.S.C. §102(b) as being anticipated by Brown. *See* Office Action, page 5. Claim 14 has been cancelled herein without prejudice to expedite prosecution of this application. Accordingly, Applicants respectfully submit that this rejection is moot.

III. Double Patenting Warning

The Office Action indicates that should claim 1 be found allowable, claim 31 will be objected to as being a substantial duplicate thereof. *See* Office Action, page 2. Applicants respectfully request reconsideration of this position, and Applicants will resolve any outstanding issue regarding this claim as appropriate.

CONCLUSION

Accordingly, Applicants submit that the present application is in condition for allowance and the same is earnestly solicited. The Examiner is encouraged to telephone the undersigned at 919-854-1400 for resolution of any outstanding issues.

Respectfully submitted,

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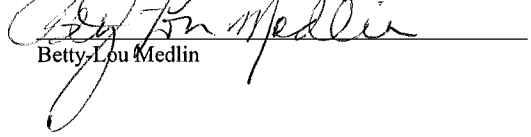

Betty-Lou Medlin

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Complete Structural Determination of Lipopolysaccharide Obtained from Deep Rough Mutant of *Escherichia coli*

PURIFICATION BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY AND DIRECT ANALYSIS BY PLASMA DESORPTION MASS SPECTROMETRY*

(Received for publication, March 7, 1988)

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Lipopolysaccharide (LPS) extracted from the deep rough mutant of *Escherichia coli* D31m4 was disaggregated with 0.1 M EDTA, pH 7.0, and fractionated on a diethylaminoethyl-cellulose column to yield the biphosphate form of LPS. After methylation, the derivative was purified by reverse-phase high performance liquid chromatography using a C_{18}-bonded silica cartridge. A linear gradient of 50–100% isopropyl alcohol/water (93:7, v/v) in acetonitrile/water (93:7, v/v) was used over a period of 60 min. The derivatized LPS showed a single major peak by high performance liquid chromatography, and this hexamethyl hexaacyl LPS was recovered and subjected to chemical analysis, plasma desorption mass spectrometry, and nuclear magnetic resonance (NMR) spectroscopy.

Chemical analysis of the purified hexamethyl LPS quantitated certain key chemical compositions. Plasma desorption mass spectrometry showed a molecular ion $(M + CH_2 + Na)^+$ at m/z 2360, which established the molecular formula and M_r to be $C_{116}H_{214}N_2O_{38}P_2$ and 2323, respectively. Thus, it contained two each of glucosamine, 2-keto-3-deoxyoctonate, and phosphate; four β-hydroxymyristates; one laurate; and one myristate. NMR spectroscopy confirmed the locations of the four ester-linked fatty acyl groups. Based on these results and the known structure of free lipid A, the complete structure of the deep-rough chemotype LPS from *E. coli* can now be presented with confidence. This is the first report of a successful purification to homogeneity and the characterization of the simplest of the LPS at the intact level. This study shows that the natural distribution of the lipid A moiety of LPS from *E. coli* D31m4 is hexaacyl/pentaacyl in a molar ratio of >90:<10. Acid hydrolysis of LPS causes the formation of the lower homologues of the free lipid A.

The outer surface of the outer membrane of Gram-negative bacteria is occupied by a complex amphipathic molecule called lipopolysaccharide (LPS)[1] (1, 2). The LPS is anchored in place by a unique hydrophobic moiety called lipid A. The complete structure of free lipid A derived from the LPS of *Salmonella* strains has been established within the past 3 years (3–7). The covalent structures of the lipid A moieties of LPS from *Escherichia coli* and the *Salmonella* strains are identical (8). This structure has now been confirmed by organic synthesis (9) and by demonstrating that the synthetic lipid A has biological activities essentially identical with those of the lipid A from natural sources (10).

The structure of the LPS molecule is presently inferred from studies done separately on the polysaccharide (11) and the free lipid A fragments or on the deacylated LPS (12, 13). A more reliable way to determine the structure of LPS would be to directly analyze a suitably purified intact LPS before degradation. But purification of LPS has been a difficult task because of the tendency of this amphipathic, anionic molecule to aggregate extensively. Detergents have been used to disaggregate the LPS before column chromatography and keep it in the disaggregated state. But, such fractionations do not produce high resolution. Moreover, it is difficult to remove the detergent from the LPS after fractionation. Detergent is not normally used in high resolution HPLC.

Previously, we successfully applied several modern instrumental methods (*viz.* ^1H NMR, ^{13}C NMR, ^{31}P NMR, fast atom bombardment mass spectrometry, and laser desorption mass spectrometry) to establish the structures of the free lipid A and lipid A precursors from Gram-negative bacteria (3, 4, 7, 14–16). As we proceeded to analyze larger amphipathic molecules such as the rough chemotype LPS, we wondered if new developments in instrumental analysis would be required. We have found that for the methylated Re-LPS, ^1H NMR, ^{13}C NMR, and PD mass spectrometry are effective. We now report the successful purification to homogeneity by HPLC of the simplest LPS obtained from the deep rough mutant of *E. coli* D31m4. The use of PD mass spectrometry was the key to characterization of this LPS at the intact level. This study has allowed us to establish the true structure of the Re-LPS and determine whether the microheterogeneity in free lipid A is due to the natural distribution or was generated by acid hydrolysis.

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[1] The abbreviations used are: LPS, lipopolysaccharide; Re-LPS, deep rough chemotype lipopolysaccharide; PD, plasma desorption; MLA, monophosphoryl lipid A; DLA, diphosphoryl lipid A; KDO, 2-keto-3-deoxyoctonate; HPLC, high performance liquid chromatography; GPC, gel permeation chromatography; DEPT, distortionless enhancement polarization transfer.

EXPERIMENTAL PROCEDURES

Materials—HPLC-grade chloroform, methanol, acetonitrile, and isopropyl alcohol were purchased from Burdick and Jackson Laboratories, Inc., Muskegon, MI. Bio-Sil HA (−325 mesh) was purchased from Bio-Rad Laboratories; Silica Gel H thin layer plates (250 μm) were purchased from Analabs, Inc., North Haven, CT. Tetrabutylammonium phosphate was purchased from Eastman, and tetrabutylammonium acetate from Fluka Chemie AG, Switzerland.

Analytical Procedures—Total phosphorus and KDO contents were determined by the methods of Bartlett (17) and Osborn (18), respectively. Samples for glucosamine assay were hydrolyzed in 3 N HCl for 4 h at 95 °C and analyzed by the method of Enghofer and Kress (19).

The sample (100 μg) for fatty acid analysis was hydrolyzed in 2.5 ml of 4 N HCl at 100 °C for 4 h. The reaction mixture was extracted three times with 3 ml of petroleum ether. The extract was dried at room temperature, methylated with diazomethane, and analyzed by gas-liquid chromatography. Analytical gas-liquid chromatography was carried out on a Packard model 428 series gas chromatograph with a glass column (3.4 m × 0.2 mm) containing 3% SP-2100 DOH, 100/200 mesh Supelcoport (Supelco, Inc., Bellefonte, PA). A flame ionization detector was used, and the injector temperature was 200 °C. The column was programmed at 4 °C/min from 150–230 °C, and the flow rate of the carrier gas (N_2) was 40 ml/min.

Growth of Bacteria and Preparation of Re-LPS—Cells of *E. coli* D31m4 were grown in a New Brunswick 28 liter fermentor at 37 °C in a LB broth medium as previously described (7). Cells were initially washed with 90% ethanol three times to remove most of the phospholipids. LPS was prepared by the method of Galanos *et al.* (20), with modification as described by Qureshi *et al.* (4).

Purification of Re-LPS—The Re-LPS (1.7 g) was suspended in 0.1 M EDTA, pH 7.0 (1 mg/ml), and sonicated for 10 min. This was stirred at 22 °C for 40 min, and 5 liters of chloroform/methanol (2:1, v/v) was added and mixed thoroughly. This mixture was centrifuged at 8000 × *g* for 10 min, and a clear lower organic layer was recovered, filtered, and evaporated to dryness on a rotary evaporator. The yield of this crude Re-LPS was 1.38 g (81%).

Fractionation of Crude Re-LPS on DEAE-cellulose Column—About 1 g of Re-LPS was applied to a DEAE-cellulose column (4.5 × 23 cm) that was packed in glacial acetic acid and washed successively with methanol and chloroform/methanol/water (2:3:1, v/v). The column was washed with chloroform/methanol/water (2:3:1, v/v) (350 ml), and the Re-LPS was eluted from the column with a linear gradient of 0.04 to 0.12 M ammonium acetate in chloroform/methanol/water (2:3:1, v/v) (2 liters). Two hundred 10-ml fractions were collected and analyzed by spot-charring on a silica gel thin layer plate. Fractions were also analyzed by thin layer chromatography using chloroform/pyridine/formic acid/water (10:12:3:1, v/v) as the developing solvent. Specific fractions were pooled and desalted with the Bligh-Dyer solvent (21). Phospholipids were found in the chloroform/methanol/water (2:3:1, v/v) wash (160 mg) and in the gradient fractions 50–60 (19.3 mg). Fractions 81–125 (102 mg) contained the major Re-LPS component as observed by thin layer chromatography. Fractions 126–200 (295 mg) were a mixture of the major component and more polar lipids.

Methylation of Re-LPS—Forty mg of fractions 81–125 was dissolved in 1.0 ml of chloroform/methanol (4:1, v/v) and passed successively through a Chelex 100 column (0.8 × 5 cm) (Bio-Rad Laboratories) and a Dowex 50 (H^+) column (0.8 × 5 cm) to yield the free acids (5, 7). Chloroform/methanol (4:1, v/v) (12 ml) was used for elution. The effluent was quickly evaporated, dissolved in 1 ml of chloroform/methanol (4:1, v/v), and immediately methylated for 2 min at 22 °C. The samples were dried under a stream of N_2 at 22 °C (7).

HPLC Fractionation of Re-LPS—HPLC was performed with two Waters 6000A solvent delivery systems (Waters Associates Inc., Milford, MA), a Waters 660 solvent programmer, a Waters U6K universal liquid chromatograph injector, a variable wavelength detector model LC-85B (Perkin-Elmer), and a radial compression module (model RCM-100, Waters Associates, Inc.).

A Nova-Pak cartridge (8 mm × 10 cm) (C_{18}-bonded end-capped 5-μ silica, Waters Associates, Inc.) was used at a flow rate of 2 ml/min. For the fractionation of hexamethyl Re-LPS, a linear gradient of 50–100% isopropyl alcohol/water (93:7, v/v) in acetonitrile/water (93:7, v/v) was used over a period of 60 min. The wavelength of the detector was set at 210 nm. The solvent system used for the fractionation of Re-LPS as the free acid was a linear gradient of 20–100% isopropyl alcohol/water (9:1, v/v) in acetonitrile/water (9:1, v/v). Both solvents contained 5 mM tetrabutylammonium phosphate or acetate.

The crude Re-LPS after the EDTA treatment was methylated, and GPC was performed. A 0.5-inch × 50-cm Jordi GPC, 500 Å (2–10 μm of polyvinylbenzene, Jordi Associates Inc., Bellingham, MA) with the solvent of hexane/isopropyl alcohol/water (53:42:5, v/v) was used at a flow rate of 1.5 ml/min. The wavelength of the detector was set at 210 nm.

Preparation of Highly Purified Hexaacyl DLA and MLA—We prepared the highly purified and well characterized hexaacyl DLA and hexaacyl MLA for use as reference standards to quantitate the phosphorus content of the HPLC-purified methylated Re-LPS.

To prepare purified DLA by the method of Rosner *et al.* (12) with modifications, the Re-LPS from *E. coli* D31m4 (0.5 g) was treated with 20 mM sodium acetate, pH 4.5 (2.5 mg/ml), at 100 °C for 90 min and extracted with chloroform/methanol (2:1, v/v) to yield 360 mg of crude DLA. The crude DLA (360 mg) was dissolved in 40 ml of chloroform/methanol (4:1, v/v) and applied to a DEAE-cellulose column (acetate form) (3.3 × 18 cm) equilibrated with chloroform/methanol/water (2:3:1, v/v). The column was first eluted with 500 ml of the solvent to yield 150 mg of unadsorbed lipid A. The column was then eluted with a linear gradient of 0.02 to 0.08 M ammonium acetate in chloroform/methanol/water (2:3:1, v/v) (21). One hundred and fifty fractions of about 13 ml were collected. The DLA fractions were detected by spot-charring 20-μl aliquots. Specific fractions were pooled, desalted with the Bligh-Dyer solvent, and dried. Fractions 51–68 (30 mg), containing the hexaacyl DLA (used as the standard), was converted to the free acid by passage through a Chelex 100 (Na^+)-Dowex 50 (H^+) double layer column in chloroform/methanol (4:1, v/v) as described for the LPS. Analytical reverse-phase HPLC and subsequent laser desorption mass spectrometry of the methylated derivative confirmed that this fraction contained the hexaacyl DLA. Later, DEAE-cellulose column fractions contained the pentaacyl and tetraacyl DLAs. A hexaacyl lipid A containing one KDO was also observed.

Re-LPS from *E. coli* D31m4 (1 g) was treated with 0.1 N HCl (150 ml) at 100 °C for 30 min as previously described (3, 4) to yield 570 mg of crude MLA. It was purified by a silicic acid (Bio-Sil HA) column according to the method of Qureshi *et al.* (7, 22) to yield the hexaacyl MLA. This was further purified by preparative thin layer chromatography as described previously (3).

PD-Mass Spectrometry—Positive ion PD mass spectra were obtained on a BIO 10N Nordic (Uppsala, Sweden) BIN-10K time-of-flight mass spectrometer, with a ^{252}Cf fission-fragment ionization source. Samples were dissolved in chloroform/methanol (1:1, v/v) saturated with the tripeptide glutathione (23) to a concentration of approximately 0.5 μg of sample per μl, and 10 μl of this sample solution was electrosprayed on the aluminum foil sample holder. Mass spectra were obtained by accumulating the ion signal to a preset value of 5×10^6 primary ion events.

Proton NMR Analysis—The ^1H NMR spectra were recorded on a Bruker AM-500 spectrometer. HPLC-purified hexamethyl hexaacyl Re-LPS was dissolved in CD_3OD, and a ^1H-^1H shift correlation experiment was conducted at 25 °C as previously described (7). ^{13}C NMR spectra were recorded on a Varian XL-300 spectrometer. The sample was dissolved in CD_3OD, and the spectrum was taken at 75 MHz using a 5-mm overall band probe. For spin-echo ^{13}C NMR analysis (24), 7000 scans were accumulated with a delay between the 90° and 180° pulses of 8 ms. Under these experimental conditions, the CH (anomeric carbon of glucosamine) and CH_3 resonances have the same phases (negative deflection) and opposite (positive deflection) to that of CH_2 and quaternary carbon (anomeric carbon of KDO). Dioxane was used as the internal standard (67.4 ppm). For the ^{13}C DEPT (distortionless enhancement polarization transfer) spectrum (25), 5600 scans were accumulated with a 3-s delay between scans. The width of the last ^1H pulse before acquisition was set to 90° so that CH and CH_3 signals have the same phase (positive deflection), whereas CH_2 signals are null. Quaternary carbons are always null in this type of experiment, regardless of the length of the last proton pulse.

RESULTS

Purification of Re-LPS—Re-LPS was extracted from *E. coli* D31m4 and treated with 0.1 M EDTA to yield the disaggregated LPS. This crude Re-LPS was fractionated on a DEAE-cellulose column to yield the partially purified Re-LPS (fractions 81-125). Analytical thin layer chromatography of the

crude Re-LPS and the column-purified Re-LPS showed that the column fractionation effectively removed all of the phospholipids and the unknown form of Re-LPS (slowest moving band). The purified Re-LPS, represented by a single band, was a mixture of the hexaacyl and pentaacyl forms and thus required further fractionation. The purified Re-LPS was methylated with diazomethane and subjected to reverse-phase HPLC using a C_{18}-bonded silica cartridge. A representative profile of such a fractionation is shown in Fig. 1. The major hexaacyl Re-LPS peak was observed at 44 min, whereas the minor pentaacyl Re-LPS was observed at 36 min.

Quantitation of several preparations of Re-LPS obtained from *E. coli* D31m4 by HPLC clearly showed that the hexaacyl form of Re-LPS is the predominant form (>90%), whereas the pentaacyl form is minor (<10%). Only trace amounts of the tetraacyl form were ever detected.

Chemical analysis of the HPLC-purified methylated LPS showed the presence of glucosamine and KDO. The phosphorus content was of special interest because it would establish whether the purified Re-LPS is a bisphosphate or a triphosphate. Thus, we were especially careful to prepare highly purified and well characterized hexaacyl MLA and DLA to use as reference standards. A precise quantitation for the phosphorus contents showed the expected phosphorus/lipid molar ratio of 0.99 and 1.99 for MLA and DLA, respectively (Table I). The purified hexamethyl hexaacyl Re-LPS gave a molar ratio of 1.99, which showed that it is indeed a bisphosphate. Gas-liquid chromatography showed that a molecule of hexaacyl Re-LPS has, as expected, four hydroxymyristates, one myristate, and one laurate.

The EDTA-treated Re-LPS was converted to the free acid and immediately methylated with diazomethane. The results

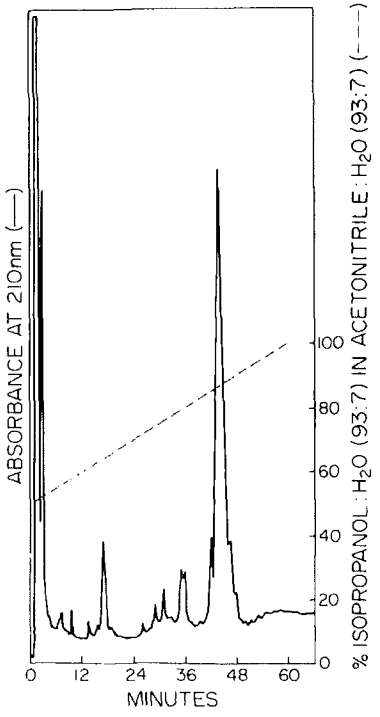

FIG. 1. **Reverse-phase HPLC of hexamethyl Re-LPS.** A C_{18}-bonded silica cartridge was used. The mobile phase was a linear gradient of 50–100% isopropyl alcohol/water (93:7, v/v) in acetonitrile/water (93:7, v/v) at a flow rate of 2 ml/min over a period of 60 min. The absorbance units at full scale was set at 0.32 for 1.6 mg of hexamethyl LPS. Baseline correction was made by the Perkin-Elmer LC-85B detector.

TABLE I

Quantitation of the phosphorus content of the HPLC-purified methylated Re-LPS

In this experiment, highly purified and well characterized MLA and DLA were used as reference standards.

Lipid	M_r	Lipid	Phosphorus[a]	Moles phosphorus/mol lipid[b]
		nmol/mg	*nmol/mg*	
Reference standard				
Hexaacyl MLA $(NH_4)_2$	1752	571	564	0.99 (1.00)
Hexaacyl DLA $(NH_4)_4$	1868	535	1064	1.99 (2.00)
Methylated Re-LPS	2323[c]	430	855	1.99 (2.00)

[a] Total phosphorus analysis was done in quadruplicate. Standard deviations did not exceed 5%.

[b] The theoretical values are given in parentheses to compare with the experimentally determined values.

[c] This M_r was calculated on the assumption that the preparation is in the hexamethyl-hexaacyl-bisphosphoryl form.

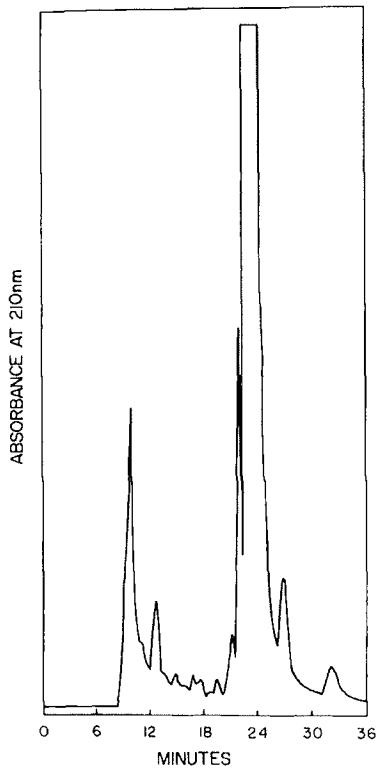

FIG. 2. **Gel permeation chromatography of hexamethyl Re-LPS.** A gel permeation chromatography column (500 Å, 2–10 μm polyvinylbenzene) was used at a flow rate of 1.5 ml/min. The mobile phase was hexane/isopropyl alcohol/water (53:42:5, v/v).

of the GPC of this sample are shown in Fig. 2. The following components were identified at the indicated elution time: 10 min, Re-LPS; 23 min, solvent peak. Interestingly, we identified a triphosphoryl Re-LPS in the leading shoulder of the bisphosphoryl Re-LPS peak at 10 min. These identifications were made by PD mass spectrometry (data not presented).

PD Mass Spectral Analysis of Hexamethyl Hexaacyl LPS—HPLC-purified hexamethyl hexaacyl LPS was analyzed by PD mass spectrometry, and the results are shown in Fig. 3. The molecular ions $(M + CH_2 + Na)^+$, $(M + 2CH_2 + Na)^+$, and $(M + 3CH_2 + Na)^+$ were observed at m/z 2360, 2373, and 2387, respectively. The extra methyl group was due to the methylation step.

Two dimethyl phosphate cleavage reactions from the re-

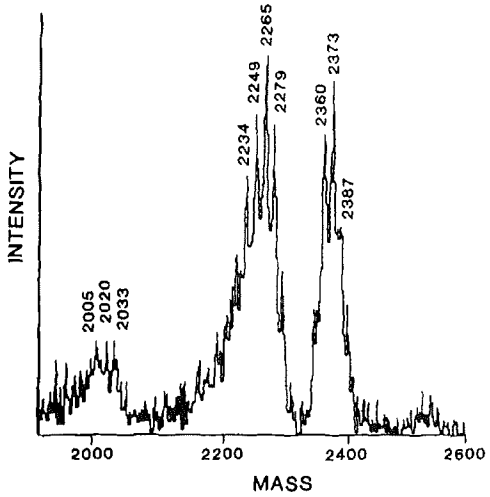

FIG. 3. **Partial PD mass spectrum of HPLC-purified hexamethyl hexaacyl Re-LPS.**

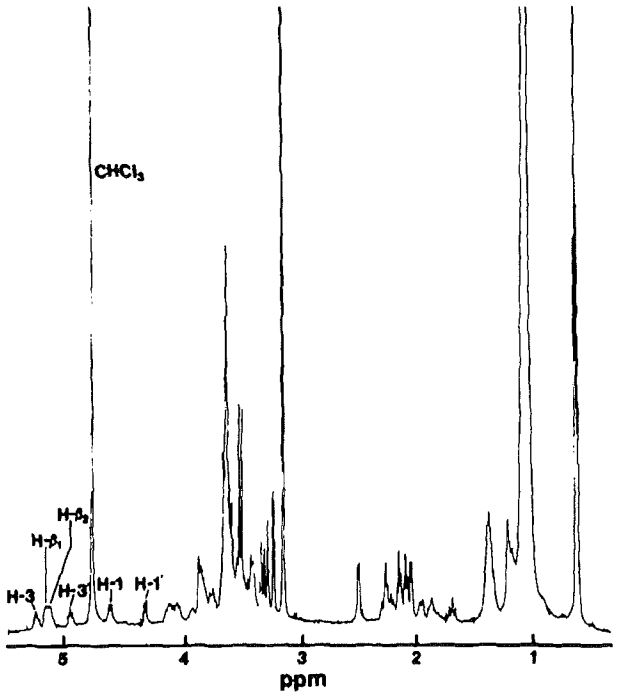

FIG. 4. **Proton NMR spectrum of the HPLC-purified hexamethyl hexaacyl Re-LPS (39 mg) in CD_3OD solution.**

ducing end were observed, and these were analogous to those observed previously by laser desorption (26). The reactions were: (i) cleavage of the O-P bond with H-transfer to the sugar (loss of $C_2H_5PO_3$); thus, $(M + 2CH_2 + Na)^+ - C_2H_5PO_3$ and $(M + 3CH_2 + Na) - C_2H_5PO_3$ ions were observed at *m/z* 2265 and 2279, respectively; and (ii) cleavage of the C_1-O bond with H-transfer from the sugar (loss of $C_2H_7PO_4$); thus, $(M + CH_2 + Na)^+ - C_2H_7PO_4$ and $(M + 2CH_2 + Na)^+ - C_2H_7PO_4$ ions were observed at *m/z* 2234 and 2249, respectively. This is consistent with the fact that the molecular ions are formed by alkali ion attachment; in this case, Na^+ (26).

The loss of the acyl-linked fatty acids was also observed. The major finding expected (and observed) was the loss of the β-hydroxymyristate from the 3-position of glucosamine disaccharide of the lipid A. $(M + 2CH_2 + Na)^+ - C_2H_7PO_4 - OHC_{14}$, $(M + 2CH_2 + Na)^+ - C_2H_5PO_3 - OHC_{14}$, and $(M + 3CH_2 + Na)^+ - C_2H_5PO_3 - OHC_{14}$ ions were observed at *m/z* 2005, 2020, and 2033, respectively. However, additional losses of laurate and myristate residues from the acyloxyacyl esters linked at the 2′- and 3′-positions may account for these poorly resolved peaks. $(M + 2CH_2 + Na)^+ - C_2H_7PO_4 - nC_{14}$ ion showed a peak at *m/z* 2033.

Based on the above results, the hexamethyl hexacyl LPS has an M_r of 2323 and contains two each of glucosamines, KDO, and phosphates; four β-hydroxymyristates; one laurate; and one myristate. One cannot exclude the possibility of additional substituents present in the Re-LPS labile to mass spectrometry.

NMR Analysis—NMR studies on the hexamethyl hexaacyl derivative were performed by ^1H and ^{13}C NMR. The initial assignment of H-1 was based upon the extra splitting of the signal at 4.62 ppm, which was attributed to the H-P coupling (spectrum not shown). The H-1′ assignment at 4.30 ppm showed a $J_{1',2'}$ of 8.5 Hz, which is consistent with the known β-anomeric configuration of the distal sugar. Several past ^1H NMR studies on similar lipids showed that H-1 is always found downfield of H-1′ (5, 7). Further assignment of the proton spectrum of methanolic solutions of HPLC-purified hexamethyl hexaacyl Re-LPS was achieved by means of one- and two-dimensional chemical shift correlation spectroscopy. A representative spectrum (Figs. 4 and 5) indicated the correlations that led to identification of the relevant protons. The correlations refer to the H-3 and H-3′ protons of the reducing end and distal glucosamine rings and the β_1 and β_2 protons of the two β-hydroxymyristates in acyloxyacyl linkage. Their downfield shifts to 5.21, 4.94, 5.13, and 5.10 ppm, respectively, from the normal 3.4–4.0 ppm for this type of protons is consistent with the presence of acyl groups at these positions. The cross-peaks for $\alpha_1\beta_1$, $\alpha_2\beta_2$, $\gamma_1\beta_1$, and $\gamma_2\beta_2$ allowed us to identify the corresponding α and γ protons in the two acyloxyacyl fatty acyl groups at the C-2′ and C-3′ positions of the glucosamine disaccharide.

The number and type of sugars in the structure of LPS were obtained by analysis of the anomeric region of its ^{13}C spectrum. Fig. 6 shows a comparison of the broad band decoupled spin-echo ^{13}C spectrum and the DEPT spectrum of LPS in CD_3OD. Four resonances at 102.2, 101.4, 99.1, and 98.2 ppm were observed in the broad band decoupled spectrum, which is consistent with the presence of four sugar units in LPS; two of these four signals, those of 99.1 and 98.2 ppm, were missing from the DEPT spectrum, indicating that they represented quaternary carbons. We attributed these two resonances to C-2″ and C-2‴ of the two KDO moieties. We have assigned the two resonances at 102.2 and 101.4 ppm to the anomeric CH of the 2 glucosamine residues.

DISCUSSION

The present study clearly demonstrates that the lipid A in the intact Re-LPS is predominantly hexaacyl (>90%). Reverse-phase HPLC was effective in separating the Re-LPS according to the number of fatty acids present in the molecule. Thus, it was the method of choice for separating the hexa-, penta-, and tetraacyl Re-LPS. By utilizing this capability of HPLC, we were able to establish the relative abundance of the hexaacyl/pentaacyl/tetraacyl Re-LPS (expressed in molar ratio) to be >90:<10:trace in *E. coli*. When this sample was hydrolyzed in 0.1 N HCl at 100 °C for 30 min and examined by HPLC as the methyl derivative, the hexaacyl/pentaacyl/tetraacyl MLA molar ratio was 56:25:20.[2] This result is con-

[2] N. Qureshi and K. Takayama, unpublished data.

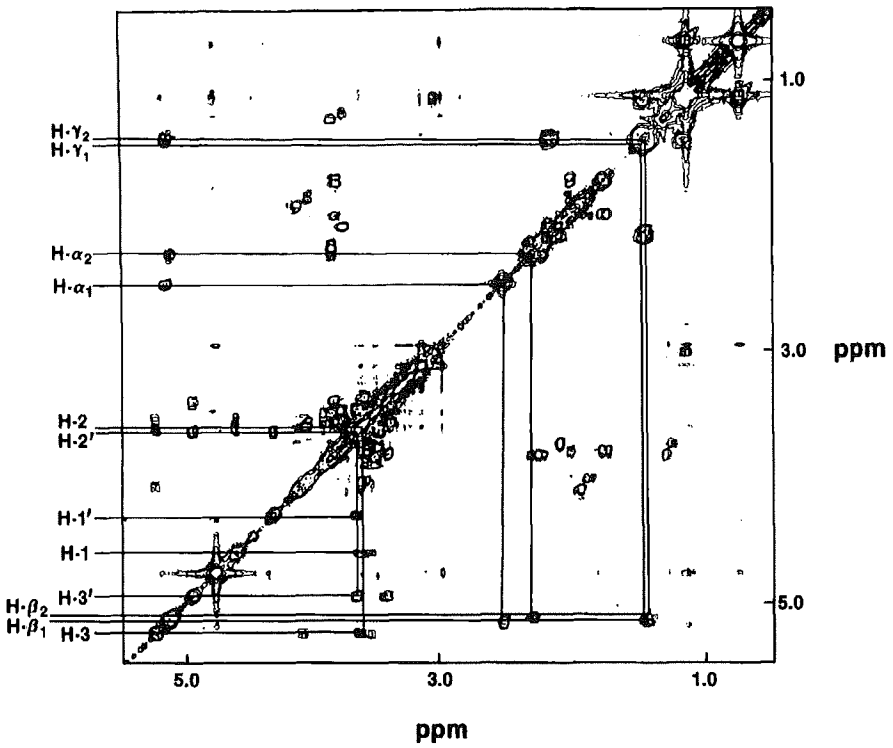

FIG. 5. **^{1}H-^{1}H shift correlation spectrum at 500 MHz of HPLC-purified hexamethyl hexaacyl Re-LPS (39 mg) in CD_3OD solution.** The contour plot shows the important scaler coupling connectivities for the protons on the two glucosamine rings and the α, β, and γ protons on the two acyloxyacyl groups.

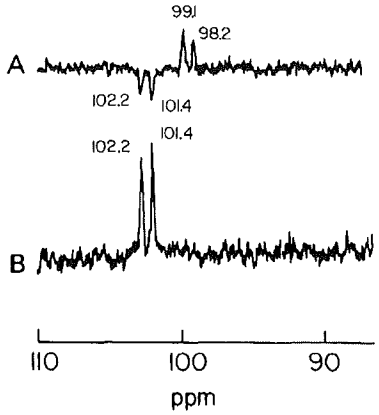

FIG. 6. *A*, spin-echo ^{13}C spectrum of hexamethyl hexaacyl Re-LPS (39 mg) in CD_3OD (0.5 ml). The spectrum was taken at 75 MHz using a 5-mm broad band probe. Dioxane (67.4 ppm) was used as the internal standard. *B*, ^{13}C-DEPT spectrum of hexamethyl hexaacyl bisphosphoryl Re-LPS in CD_3OD, same region as in *A*.

sistent with all previous studies, where heterogeneity of "free" lipid A preparations has been reported (3, 4, 7, 27), and hexaacyl lipid A has been the most prominent component (4, 7). Thus, it is now clear that acid-catalyzed hydrolysis of hexaacyl and pentaacyl Re-LPS causes the release of labile fatty acid groups, primarily at two ester-linked positions, to yield free lipid A that contains a greater abundance of the pentaacyl and tetraacyl forms. In our study, very little, if any, of the diacyl and triacyl MLA were formed. Our results were not consistent with those obtained by Batley *et al.* (28) who claimed that the average fatty acid content of the LPS from *Salmonella minnesota* R595 is four. This difference in the two results might reflect the quality of the respective preparations studied.

Most of the structural and biological studies have been performed with LPS preparations that differ in their degree of purity. Currently, electrodialysis (29), sodium dodecyl sulfate-polyacrylamide gel electrophoresis (30–32), and gel filtration in the presence of detergents (33, 34) are the methods of choice for the purification of the LPS (from smooth strains). We have developed a simple and nondestructive method to purify and characterize the simplest of LPS (Re) at the intact level. LPS obtained by the Galanos method (20), as modified by Qureshi *et al.* (4), was disaggregated using a concentrated EDTA solution as the chelating agent. It was then extracted into the chloroform/methanol phase using the Bligh-Dyer method (21). EDTA was previously used by Leive (35) to release up to 50% of the LPS from whole cells. The effectiveness of EDTA in removing the divalent cations in LPS was illustrated by the study of Coughlin *et al.* (36). They dialyzed the electrodialyzed LPS against EDTA solution to remove the remaining tightly associated divalent cations (29, 36) and replaced them with sodium.

The disaggregated LPS, now soluble in chloroform/methanol, was purified by DEAE-cellulose column chromatography using an ammonium acetate gradient in chloroform/methanol/water (2:3:1, v/v). This fractionation not only removed all the phospholipids but also separated the bisphosphoryl Re-LPS from the other forms, including the triphosphoryl Re-LPS. This highly purified Re-LPS is suitable for biological studies because it is essentially devoid of non-LPS lipids. After converting to the triethylammonium salt, this preparation forms a clear suspension in water.

The methylated LPS was fractionated further by reverse-phase HPLC. For this separation, we modified the acetonitrile/isopropyl alcohol solvent system that was previously developed for MLAs (7). Optimal separation required the addition of 7% water to the organic solvent system, which presumably was due to the additional presence of the polar KDO units in the Re-LPS molecule. The underivatized Re-LPS could also be fractionated by this method, provided the

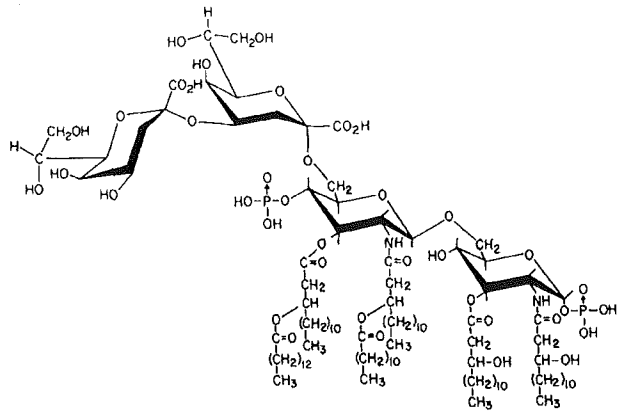

FIG. 7. **Proposed structure of the hexaacyl bisphosphoryl Re-LPS found in the deep rough mutant of *E. coli* D31m4.**

tetrabutylammonium salt was added to the solvent. We have now demonstrated that we can purify intact LPS by HPLC to homogeneity. The important point to be made here is that such a purified intact molecule is now amenable to modern instrumental analysis (NMR and mass spectrometry). The method described here might be applicable to the eventual fractionation and characterization of more complex lipopolysaccharides (Rd, Rc, Rb, and Ra chemotypes).

Previous studies of the structure of MLA from *Neisseria gonorrhoeae* (37) and *E. coli* (23) using laser desorption mass spectrometry showed prominent fragment ions resulting from the simultaneous cleavage of the C_3-C_4 and C_1-O bonds of the reducing end glucosamine, with the charge remaining on the distal portion. The analogous cleavage was also observed in the laser desorption mass spectrum of the methylated Re-LPS of *E. coli* as previously reported (26). In that study, the measured mass (1681 atomic mass units) was 468 mass units above that observed in the free DLA. These results supported the belief that the two KDO units are attached to the distal subunit.

^{13}C NMR analysis confirmed the presence of the two KDO units that exist as a tautomeric mixture and are attached to the primary hydroxyl group at the 6′ position of the glucosamine disaccharide of the lipid A moiety. These results were consistent with those of Brade and Rietschel (38) and Zahringer *et al.* (39). The linkage between the two KDO is 2→4 (38–40).

Based on this study, we have now established the precise structure of the major Re-LPS component found in *E. coli* D31m4, as shown in Fig. 7. It is satisfying to note that this structure is essentially identical with the structure determined by the separate analysis of the various hydrolysis products of the Re-LPS. They include the analyses of the free lipid A (4–7), the *O*-deacylated Re-LPS and lipid A (12, 13, 39), and the liberated KDO units (38, 40). However, this study has shown that there are several other prominent unidentified structural forms of Re-LPS that are more polar than the above mentioned hexaacyl bisphosphoryl Re-LPS, and these include the triphosphoryl Re-LPS. Thus, the Re-LPS of *E. coli* is a heterogeneous mixture, but all we know is the structure of the major component. We are now investigating the nature of this microheterogeneity in the Re-LPS of *E. coli*.

Finally, it is now possible to obtain a highly purified and fully characterized LPS (the hexaacyl-pentaacyl-bisphosphoryl Re-LPS) for use in biological studies.

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